Serotonin and Nitric Oxide Regulate Metamorphosis in the Marine Snail *Ilyanassa obsoleta*

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Leise, E.M., Thavaradhara, K., Durham, N. R., and Turner, B.E. (2001) Serotonin and Nitric Oxide Regulate Metamorphosis in the Marine Snail *Ilyanassa obsoleta*. *American Zoologist* 41:258-267.

Synopsis:

Several neuroactive compounds have been implicated as playing roles in the circuitry that controls larval metamorphosis in marine molluscs. For the caenogastropod *Ilyanassa obsoleta,* results of neuroanatomical studies suggest that the production of nitric oxide (NO) increases throughout the planktonic stage and that NO production is necessary for the maintenance of the larval state, especially as it becomes metamorphically competent. Bath application or injection of exogenous serotonin (5HT) can initiate metamorphosis in competent larvae, and exogenous NO can inhibit such serotonergically-induced metamorphosis. Inhibition of endogenous nitric oxide synthase (NOS) can also trigger larval metamorphosis. The production of endogenous NO appears to decrease concurrently with the initiation of metamorphosis, but the specific interactions between serotonergic and nitrergic neurons are unknown. Evidence in support of NO acting to up-regulate the enzyme guanylyl cyclase (GC) is still equivocal. Thus, we do not yet know if NO exerts its effects through the actions of cyclic 3',5'-guanosine monophosphate (cGMP) or by a cGMP-independent mechanism. The ubiquity of nitrergic signal-ling and its significance for developing molluscan embryos and larvae are still the subject of speculation and require further investigation.

Article:

INTRODUCTION

Investigations into the cellular circuitry and biochemical pathways that underlie molluscan metamorphosis are relatively few when compared to the literature for metamorphosis in the terrestrial arthropods. The long generation times, seasonal reproductive strategies and relatively small sizes of molluscan larvae and young juveniles have made them difficult experimental organisms. As a result, our understanding of the mechanisms by which molluscan metamorphosis is regulated is limited. Despite these drawbacks, in the last 25 yr, a few molluscan species have been used to examine this important developmental event from a mechanistic perspective. Metamorphosis has probably been most well studied in the nudibranch *Phestilla sibogae* (Had-field and Karlson, 1969; Hadfield, 1977, 1978, 1998, Pires *et al.,* 1997, 2000a), the abalone *Haliotis rufescens* (Morse *et al.,* 1979; Trapido-Rosenthal and Morse, 1985, 1986a, *b;* Morse, 1990; Wodicka and Morse, 1991; Baxter and Morse, 1992; Degnan *et al.,* 1997), and in the oysters *Crassostrea virginica* and C. *gigas* (Bonar *et al.,* 1990; Coon *et al.,* 1990a, *b;* Zimmer-Faust and Tamburri, 1994; Beiras and Widdows, 1995). Studies on these and other species have indicated that commonly occurring neurotransmitters, neuromodulators, and second messenger pathways play important roles in the control of metamorphosis. When competent, or capable of undergoing metamorphosis, molluscan larvae share certain characteristics, but the physiological interactions that govern the transformations of metamorphosis may be as varied as their life histories. In this paper, to put our investigations into context, we summarize some of the relevant work on the biochemical pathways that are active during the initiation of metamorphosis. With apologies to those whose work we might leave out, we are still far from having a complete understanding of how the transformations of metamorphosis are controlled in even one species.

Competent, free-swimming veliger larvae share several key features. They possess distinctive velar lobes, the larval swimming and feeding structures, they have active nervous systems (NSs) containing rudiments of most

or all of their adult ganglia, and each larva possesses an apical sensory organ (Kume and Dan, 1968; Lacalli, 1994; Croll *et al.*, 1997; Marois and Carew, 1997a, *b).* Loss of the velum is characteristically the first visible sign of metamophosis, and where it has been examined, the cephalic or apical sensory organ (ASO) is lost by the end of this process (Bonar and Hadfield, 1974; Bonar, 1978b; Lin and Leise, 1996a; Marois and Carew, 1997b). ASOs typically contain 3-5 serotonergic neurons and a number of putative sensory neurons (Kempf *et al.*, 1997; Marois and Carew, 1997a, *b, c;* Page and Parries, 2000). In some gastropods, the ASO is part of a distinct apical ganglion (AG) that contains 2-3 clusters of neuronal somata and a subjacent neuropil (Leise, 1996; Lin and Leise, 1996a; Kempf *et al.*, 1997; Marois and Carew, 1997a, *b, c;* Page and Parries, 2000). Chemosensory neurons that respond to metamorphic inducers have been postulated to occur within the ASO (Bonar, 1978a; Chia and Koss, 1982,1984; Kempf *et al.*, 1997; Marois and Carew, 1997a). A series of experiments on abalone larvae, including the isolation of signal transduction molecules from epithelial cilia and related behavioral and receptor binding studies (Trapido-Rosenthal and Morse, 1986a, *b;* Wodicka and Morse, 1991; Baxter and Morse, 1992), and experimentation on DASPEI-dependent fluorescence of epidermal sensory cells in *Phestilla* (Hadfield, 1998; Hadfield *et al.*, 2000), provide strong support for the idea that cells of the ASO can perceive metamorphic stimuli. Regions of the larval foot have also been suggested as potential sites for chemosensory activities in the nudibranch *Onchidoris bilamellata* (Arkett *et al.*, 1989; Chia and Koss, 1989).

How sensory information might be integrated within the larval NS and then used to activate appropriate target tissues is still the subject of speculation. From a behavioral standpoint, we know that pre-competent nudibranch and abalone larvae can habituate to an inducer substance and show decreased rates of metamorphosis when competent (Hadfield, 1980; Hadfield and Scheuer, 1985; Trapido-Rosenthal and Morse, 1986a, *b).* However, these experimental results can most easily be interpreted as interactions occurring at the level of individual receptor cells (Hadfield and Scheuer, 1985; Trapido-Rosenthal and Morse, 1986a, *b).* Studies of gene expression and downstream networks in competent and metamorphosing molluscan larvae are also in their infancy, but recent studies suggest that metamorphic pathways may be highly variable. For example, even in the presence of inhibitors of protein synthesis, *P. sibogae* can proceed through metamorphosis, but new juveniles are unable to undergo elongation (Hadfield, 1998). Conversely, studies on *H. rufescens* have demonstrated that these gastropods cannot progress through the final stages of metamorphosis if translation is blocked (Fenteany and Morse, 1993). Degnan *et al.* (1997) have identified at least one homeobox gene in abalone whose expression decreases throughout larval development and then transiently increases during metamorphosis. But again, how changes in gene expression and cellular protein levels are triggered once a larva encounters a metamorphic inducer remain to be determined.

Numerous neuroactive compounds, including γ-aminobutyric acid (GABA), various catecholamines, serotonin (5HT), and nitric oxide (NO) are associated with metamorphic processes in a variety of molluscs. GABA can induce metamorphosis in abalone (Morse *et al.*, 1979), in the conch *Strombus gigas* (Boettcher and Targett, 1998) and in the nudibranch *Hermissenda crassicornis* (Avila *et al.*, 1996). For *Hallotis,* exogenous GABA mimicks components of the natural algal inducer and acts as an external ligand, rather than as an internal neurotransmitter (reviewed in Morse, 1990). Its mode of action in *Strombus* and *Hermissenda* is unknown. Similarly, choline chloride can induce metamorphosis in nudibranch larvae, and while it is presumed to be acting as part of an internal cholinergic pathway, its mode of action is also unresolved (Hirata and Hadfield, 1986; Todd *et al.*, 1991; Avila *et al.*, 1996).

A variety of catecholamines, including epinephrine (EP), norepinephrine (NE), dopamine (DA), and L-3,4-dihydroxyphenylalanine (DOPA), have been implicated as inducing or being necessary for the induction of metamorphosis in several molluscs. In oysters, exogenous DOPA can trigger settlement behavior after conversion to DA within the larvae, whereas adrenergic molecules are necessary for the induction of the morphogenetic aspects of metamorphosis (reviewed in Bonar *et al.*, 1990; Beiras and Widdows, 1995). Exogenous catecholamines, such as NE and EP, can induce significant amounts of metamorphosis in *Strombus* (Boettcher and Targett, 1998), but in this case, larvae may be responding to breakdown products, including dissolved hydrogen peroxide, as was suggested to be the case for partial metamorphosis induced by a number of

catecholamines in *P. sibogae* (Pires and Hadfield, 1991). However, direct measurement by high performance liquid chromatography of endogenous catecholamines in competent and metamorphosing larvae of *P. sibogae* and the slipper limpet *Crepidula fornicata* have revealed that levels of DOPA, DA, and NE increase during larval development (Pires *et al.,* 1997, 2000b). Experimental depletion of NE and DA in *Phestilla* and of DOPA and DA in *Crepidula* results in inhibition of naturally-induced metamorphosis (Pires *et al.,* 1997, 2000b). Exposure of competent *Phestilla* to DOPA also potentiates the activity of the natural coral inducer (Pires *et al.,* 2000a). These findings, combined with the immunocytochemical localization of catecholamines in the central nervous systems (CNSs) of larval *Phestilla* (Kempf *et al.,* 1992), demonstrate both the metamorphic dependence upon catecholamines by nudibranch larvae and the necessity for experimentation that extends beyond simple bath application of neurotransmitters.

In our laboratory, we study metamorphosis in the prosobranch gastropod *Ilyanassa obsoleta* for several reasons. Egg capsules can be obtained throughout the year from breeding populations of adults, and larval culture is likewise routine (Leise, 1996). Like *Phestilla* and *Haliotis, Ilyanassa* is specific in its metamorphic requirements, responding to water-soluble extracts of sediment from intertidal mudflats (Scheltema, 1961). Recently, we obtained a natural inducer substance from cultures of the tychopelagic centric diatom *Coscinodiscus* sp. (Leise *et al.,* 1996). Previously, Levantine and Bonar (1986) discovered that exogenous 5HT would initiate metamorphosis in competent *Ilyanassa,* so we can now induce this species to metamorphose at will (Couper and Leise, 1996). In general, we are concerned with the cellular events that link the morphological and physiological changes of metamorphosis to the neural reception of a stimulating cue. Our studies are far from complete, but to date, our findings have allowed us to raise testable hypotheses about neural interactions that might occur during the induction of this developmental phenomenon.

SEROTONIN, EMBRYOS, LARVAE, AND METAMORPHOSIS

Serotonergic neurons arise early in gangliogenesis in pulmonate (Marois and Croll, 1992; Dickinson *et al.,* 1999), prosobranch (Barlow and Truman, 1992), and opisthobranch (Marois and Carew, 1997b) embryos. Serotonin regulates neurite outgrowth during development in the pulmonate *Helisoma trivolvis* (Goldberg and Kater, 1989) and increases the frequency of ciliary beating in these embryos and in several nudi-branchs (Koshtoyants *et al.,* 1961; Gold-berg *et al.,* 1994). As mentioned, serotonergic neurons are a characteristic feature of molluscan ASOs (Kempf *et al.,* 1997; Marois and Carew, 1997a, *b, c;* Dickinson *et al.,* 1999) and similar structures across the animal phyla (reviews in Lacalli, 1994; Marois and Carew, 1997a). *Larval Ilyanassa* are no exception and possess 5 serotonergic neurons in their apical ganglia (S. C. Kempf and E. M. Leise, unpublished data). In veligers, serotonergic fibers are widespread, innervating the velum, various muscle groups, viscera and other neurons of the CNS (Kempf *et al.,* 1997; Marois and Carew, 1997c), but the functions of these serotonergic neurons have not been directly explored. Serotonin is a weak inducer of metamorphosis in *C. gigas* (Beiras and Widdows, 1995) and *Hermissenda* (Avila *et al.,* 1996), but is remarkably effective in *Ilyanassa,* inducing 80-100% of competent larvae to metamorphose in 24-48 hr (Fig. 1).

Although the strength of *Ilyanassa's* response to 5HT was clear, Levantine and Bonar (1986) did not determine whether exogenous 5HT was acting internally as a neurotransmitter or externally as a ligand, as GABA does for *Haliotis* (Morse *et al.,* 1979). Through a series of pharmacological experiments, Couper and Leise (1996) provided support for the former idea, that exogenous 5HT was acting internally to modulate larval functions. Injections of fluoxetine, a 5HT reuptake inhibitor which potentiates synaptic actions of 5HT, induced significant levels of metamorphosis (Fig. 1A), as did α-methy1-5HT, a 5HT agonist. As expected, injections of gramine, a 5HT antagonist (Fig. 1B), reduced levels of 5HT-induced metamorphosis (Couper and Leise, 1996). Thus, in at least *Ilyanassa,* serotonergic neurons appear to be active in the metamorphic pathway. Whether these injections mimicked actions of the serotonergic neurons in the apical ganglion is unknown. It is also intriguing, given the apparent ubiquity of larval serotonergic neurons, that *Ilyanassa is* still one of the few molluscan larvae to show such a strong response to this compound.

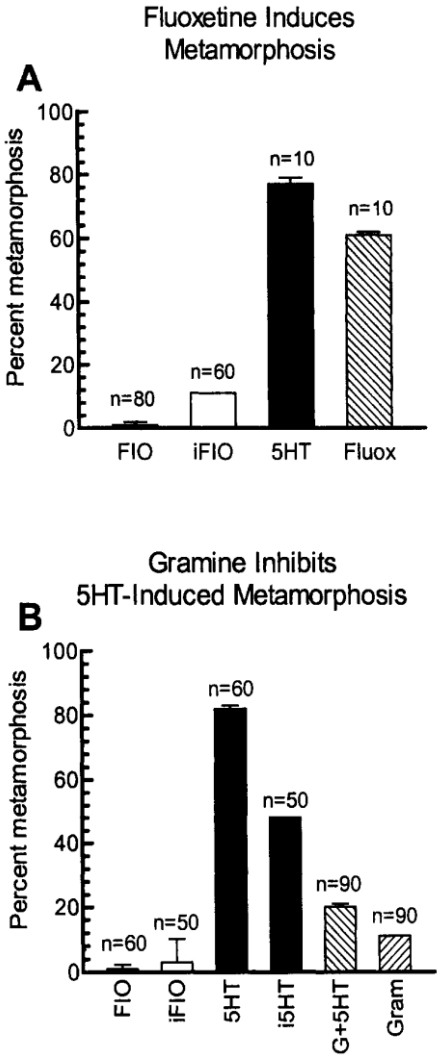

FIG. 1. Graphs of mean percentage (± SD) of metamorphosed larvae 48 hr after treatment. Only competent larvae were used in these experiments. Injected larvae were placed in 0.2 μm filtered Instant Ocean (FIO) after treatment. In each experiment, controls included a group of uninjected larvae in FIO, which usually induced <5% metamorphosis, and a group exposed to 10^{-4} *M* 5HT (5HT) in FIO which usually induced >80% metamorphosis. Additionally, controls included a group of larvae injected with FIO (iFIO) and another injected with 10^{-4} *M* 5HT (i5HT). (**A**) Injections of fluoxetine (Fluox), the 5HT re-uptake inhibitor, at 10^{-6} *M*, induced 61% metamorphosis. Statistical comparison of Fluox and iFIO using Chi Square contingency tables showed a highly significant effect. Fluox was not significantly different from 5HT ($\chi^2_{0.01(1)} = 2.42$, accept H_o). (**B**) Injections of the 5HT antagonist gramine (Gram) at 10^{-4} *M* reduced 5HT-induced metamorphosis from 48% to 20%. A 3-way Chi-Square analysis comparing gramine, injections of gramine plus 5HT (G+5HT), and 5HT alone showed that this reduction is significant ($\chi^2_{0.01(2)} = 26.27$, graphs reproduced from Couper and Leise, 1996).

NITRIC OXIDE AND METAMORPHOSIS

NO is often expressed transiently during development and effects may appear contradictory depending upon the type and concentration of NO-donor used and the cells being examined (Van Wagenen and Rehder, 1999). For example, NO released from 1-2 mM solutions of the NO-donor 3- morpholino-sydnonimine (SIN-1) mediates growth cone collapse in frog retinal ganglion cells (Renteria and Constantine-Paton, 1996) and rat dorsal root ganglion cells (Hess *et al.*, 1993), but lower concentrations *(i.e.*, 150 μM) enhance filopodial ex-tensions from growth cones of *Helisoma* neurons (Van Wagenen and Rehder, 1999). NO also appears to regulate synaptogenesis in insect peripheral and central NSs (Truman *et al.*, 1996; Ball and Truman, 1998; Gibbs and Truman, 1998; Wright *et al.*, 1998), at vertebrate neuromuscular junctions (Wang *et al.*, 1995) and retinotectal synapses (Wu *et al.*, 1994). NO also regulates the switch from cell growth to differentiation in insect and vertebrate neurons (Peunova and Enikolopov, 1995; Kuzin *et al.*, 1996).

By comparison with the above studies, actions of NO in developing molluscs have received relatively little investigation. Nitric oxide synthase (NOS) activity, as demonstrated by the use of NADPH diaphorase (NADPHd) histochemistry, occurs in the NSs of larval and juvenile *Ilyanassa* (Lin and Leise, 1996b), in similar stages in the pond snail *Lymnaea stagnalis* (Serfozo *et al.*, 1998), and in larval *P. sibogae* (Meleshkevitch *et al.*, 1997). In *Ilyanassa*, NADPHd activity occurred in all ganglia, increased throughout larval development, and was most intense in the neuropil of the AG. NADPHd activity decreased dramatically during metamorphosis (Fig. 2) followed by the emergence of a juvenile pattern of staining (Lin and Leise, 1996b). These results were consistent with 3 possibilities: (1) that NO was an endogenous inhibitor of metamorphosis in larvae, being

necessary for the maintenance of the larval state, (2) that high tissue levels of NO were necessary for the induction of metamorphosis, or (3) that NO had no effect on metamorphosis per se, being active in other larval and juvenile circuits. To distinguish between these possibilities, Froggett and Leise (1999) conducted a series of pharmacological experiments using reagents that affected nitrergic activities. Because NO diffuses through tissue, NO-donors, such as SIN-1 or S-nitroso-N-acetyl-D, L-penicillamine (SNAP), need no injection to ensure the movement of NO across the larval epithelium. Alone, neither SIN-1 nor SNAP (Fig. 3A) had any effect on comp-tent larvae (Froggett and Leise, 1999). However, when applied in conjunction with 5HT, 1 mM and 0.1 mM SNAP reduced rates of 5HT-induced metamorphosis (Fig. 3B, Froggett and Leise, 1999). Degassed solutions of NO-donors, which are made up 72 hr in advance of the experiment and retain only the soluble byproduct, showed no activity, indicating the nitrergic specificity of our results. Injections of NOS inhibitors, such as N-methyl-L-arginine acetate (L-NMMA) or N-nitro-L-arginine methyl ester (L-NAME, Fig. 4), in the absence of any inducer substance, triggered significant levels of metamorphosis, suggesting that NOS activity was necessary for the maintenance of the larval condition, but was inhibited during metamorphosis (Froggett and Leise, 1999).

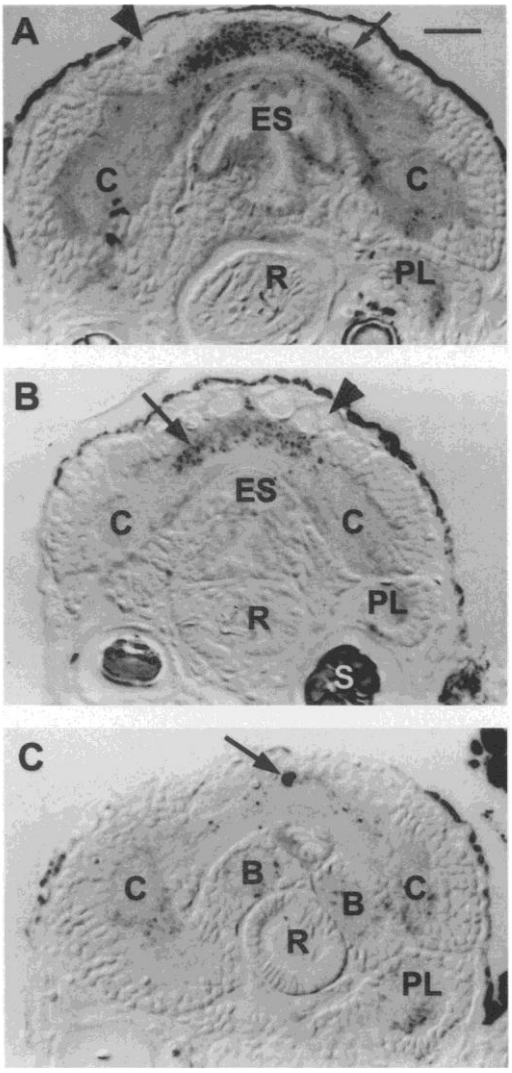

FIG. 2. Transverse, 8 μm plastic sections through the apical and cerebral ganglia of a competent larva (**A**), a metamorphosing larva (**B**), and a newly metamorphosed juvenile (**C**) of *Ilyanassa obsoleta*. Specimens were all processed for NADPH diaphorase histochemistry. (**A**) Note dark, punctate reaction product in neuropil (arrow). Large cells (arrowhead) above neuropil are somata of neurons in the apical ganglion. The neuropil lies atop the commissure connecting the two cerebral ganglia (C). (**B**) Decrease in intensity of the reaction product (arrow) occurred in the neuropil of the apical ganglion as the larva began metamorphosis. At this time, the cells of the AG (arrowhead) shifted towards the center of the larva to become one continuous group. (**C**) Newly metamorphosed juveniles, at 2 days post-induction, contained remnants of the apical ganglion atop the cerebral commissure, but the ganglionic neuropil expressed little NADPH diaphorase activity. The dark spot of reaction product (arrow) is in neuropil, not a neuronal soma. B, buccal ganglion; ES, esophagus; PL, pleural ganglion; R, radula; S, statocyst. Scale bar = 20 μm for all micrographs (all micrographs modified from Lin and Leise, 1996*b*).

The most common target for NO is soluble guanylyl cyclase (GC), the enzyme that produces cGMP (Murad *et al.,* 1978; Ignarro *et al.,* 1987). At present, we are conducting experiments to determine if larval NOS activity is cGMP-dependent. Experiments with phosphodiesterase and GC inhibitors, and results of enzyme immunoassays, suggest that larval NO activity may depend upon cGMP (Durham *et al.,* 2000), but our results are not yet conclusive. It is possible that the nitrergic activities occurring in larval *Ilyanassa* do not depend on GC

activity, a situation that occurs in other experimental systems. As examples, NO has been found to inhibit calcium channels in glomus cells of rabbit carotid bodies (Summers *et al.*, 1999) and activate a potassium current in vertebrate olfactory receptor cells (Schmachtenberg and Bacigalupo, 1999), both without any apparent activation of GC.

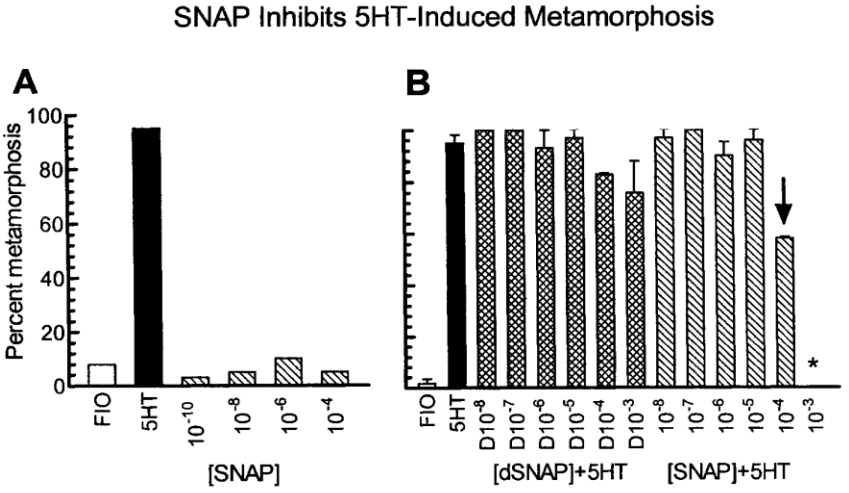

FIG. 3. (**A**) Application of the NO-donor SNAP to competent larvae induced no significant levels of metamorphosis by 48 hr. Bath application of SNAP without 5HT induced rates of metamorphosis similar to those induced by FIO. Concentrations of SNAP $>10^{-4}$ *M* produced abnormal larval behavior and decalcification but no loss of velar tissue or any indication of metamorphosis. N = 40 for each treatment. (**B**) Application of SNAP inhibited 5HT-induced metamorphosis at 48 hr. Asterisk indicates concentration of SNAP that significantly inhibited 5HT-induced metamorphosis at 24 and 48 hr. Arrow indicates concentration that was inhibitory only at 24 hr (40% metamorphosis in 10^{-4} *M* SNAP + 5HT compared to 77% in 5HT, $\chi^2_{0.005(1)} = 11.3$), but not at 48 hr. Solutions of SNAP have a half-life of about 1 hr and were changed every 6 hr to maintain relatively steady concentrations of NO. D10^{-x} = degassed solution of 10^{-x} *M* SNAP plus 10^{-4} *M* 5HT ([dSNAP] + 5HT); 10^{-x} = active solution of 10^{-x} *M* SNAP plus 10^{-4} *M* 5HT ([SNAP] + 5HT). N = 30 for each treatment (graphs modified from Froggett and Leise, 1999).

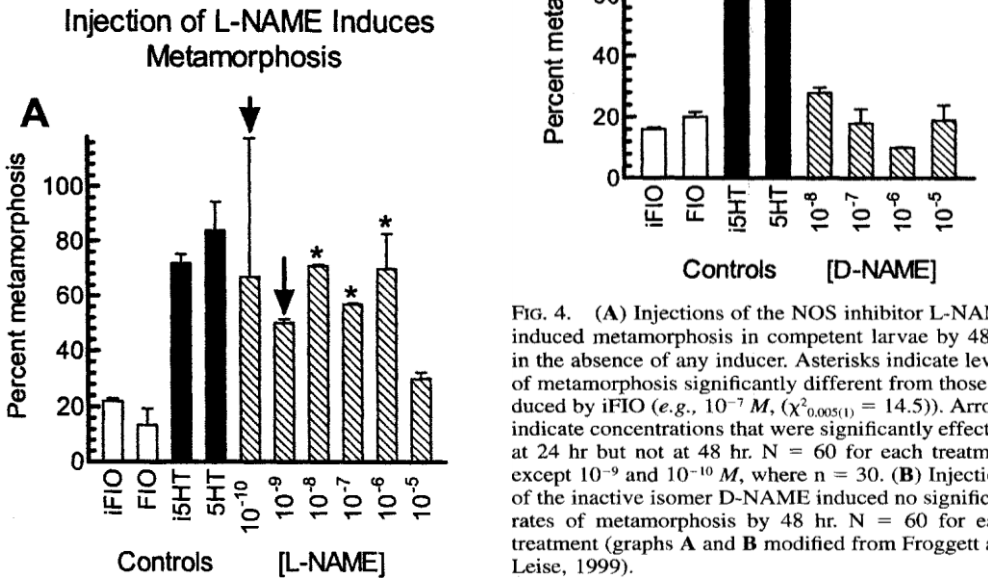

FIG. 4. (**A**) Injections of the NOS inhibitor L-NAME induced metamorphosis in competent larvae by 48 hr in the absence of any inducer. Asterisks indicate levels of metamorphosis significantly different from those induced by iFIO (*e.g.*, 10^{-7} *M*, ($\chi^2_{0.005(1)} = 14.5$)). Arrows indicate concentrations that were significantly effective at 24 hr but not at 48 hr. N = 60 for each treatment except 10^{-9} and 10^{-10} *M*, where n = 30. (**B**) Injections of the inactive isomer D-NAME induced no significant rates of metamorphosis by 48 hr. N = 60 for each treatment (graphs **A** and **B** modified from Froggett and Leise, 1999).

In addition to its mode of action, we also want to understand the cellular functions of NO in larval *Ilyanassa.* NO can trigger excitotoxic cell death following neural injury or trauma, but can also act endogenously to protect cells against premature apoptosis (Kim *et al.*, 1997; Liu and Stainler, 1999). The localization of NOS immunoreactivity to cells of the AG (Thavaradhara *et al.*, 1999) suggests the possibility that NO may protect these cells from apoptotic loss until they are no longer needed by the juvenile snails.

NITRIC OXIDE AND SEROTONIN INTERACTIONS

The decrease in NADPHd staining seen in metamorphosing *Ilyanassa* followed serotonergically induced metamorphosis. To date, the most parsimonious explanation for our results is that excitation of serotonergic sensory neurons in the AG inhibits NOS activity in postsynaptic nitrergic ones when a larva detects an appropriate metamorphic cue. As a modulator of neural functions, 5HT receptors activate several second messenger pathways which can include activation of the enzymes adenylyl cyclase and phospholipase C (Weiger, 1997). Intracellular signalling molecules could ultimately be involved in regulating NOS activity by phosphorylation or by inducing changes in cellular levels of Ca^{2+} and calmodulin (Dawson and Dawson, 1996).

Whether or not the serotonergic neurons in the AG are nitrergic, NO can directly interact with 5HT to form dimers and nitro- and nitroso-5HT, all of which are inactive at established serotonergic modulatory sites in at least one well identified synapse in the sea hare *Aplysia californica* (Fossier *et al.*, 1999). NO can also reduce levels of 5HT by inactivating tryptophan hydroxylase (TH), the rate-limiting enzyme that begins the formation of 5HT (Kuhn and Arthur, 1997). Thus, the reduction in rates of 5HT-induced metamorphosis reported by Froggett and Leise (1999) for NO-donors may have resulted from a direct inactivation of 5HT or TH. However, the induction of metamorphosis seen with injections of NOS inhibitors suggests that the actions of the NO-donors were not experimental artifacts. In competent larvae, endogenous NO could inactivate TH to inhibit 5HT production, but we more reasonably argue that NO has another function, per-haps acting to protect the neurons of the AG from premature apoptosis. This protective function would then be discontinued once 5HT neurons are excited. Clearly, more work is necessary to determine if this is the case. However, we are hopeful that as we obtain further information about the control and co-ordination of metamorphosis in *Ilyarzassa*, we will be elucidating mechanisms that have been retained by a broad array of molluscan species.

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